PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12M 1/18 | A1

(11) International Publication Number: **WO 99/55826**

(43) International Publication Date: 4 November 1999 (04.11.99)

(21) International Application Number: PCT/US99/08684

(22) International Filing Date: 21 April 1999 (21.04.99)

(30) Priority Data:
09/065,776 24 April 1998 (24.04.98) US

(71) Applicant: GENOVA PHARMACEUTICALS CORPORATION [US/US]; 4233 Town Court North, Lawrenceville, NJ 08648 (US).

(72) Inventors: CEN, Hui; 5142 Masonic Avenue, Oakland, CA 94618 (US). SUN, Shaojian; 4233 Town Court North, Lawrenceville, NJ 08648 (US).

(74) Agents: ANTLER, Adriane, M. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

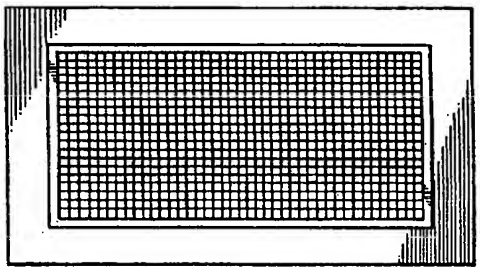
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

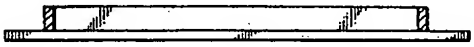
(54) Title: MICRO–COMPARTMENTALIZATION DEVICE AND USES THEREOF

(57) Abstract

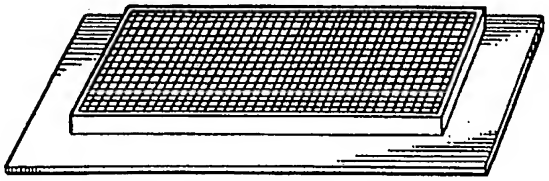
This invention relates to designs of an apparatus having a base and a removable grid to create micro–compartments. The apparatus can be used to create local, high concentrations of viruses, microbials, secretable factors and/or other biologics produced from a source of interest for biological screening (i.e. functional gene screening, secreted factor screening, etc.). The apparatus can also be used in high throughput drug screening procedures, especially in such procedures using cell–based assays, so as to avoid non–homogeneity of assay conditions and to reduce readout noise. This apparatus provides a means for parallel automated screening.



A



B



C

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

MICRO-COMPARTMENTALIZATION DEVICE AND USES THEREOF

1. FIELD OF THE INVENTION

The present invention relates generally to an apparatus, and methods for its use, to carry out massively parallel screening for biological and/or drug discovery. More particularly, the present invention relates to an apparatus and methods for signal enhancement and parallel assay in an array format in cell-based and/or biochemical-based systems used for gene discovery, gene function discovery, and/or pharmaceutical screening. This invention provides a method to enhance the ratio of a biological readout signal to background noise. This invention further provides a method which enables automated, parallel assaying in a high throughput format for biological and pharmaceutical applications.

2. BACKGROUND OF THE INVENTION

The availability of various bioassays enables the study of biological functions and biochemical properties of particular proteins, nucleic acids and/or other molecules, whether such molecules are in the form of pure components or mixtures of components. Such bioassays can measure enzymatic activity, post-translational modifications (such as degradation, glycosylation, phosphorylation, *etc.*), and/or changes in the quantity of a particular molecule (*e.g.* a protein, a polypeptide or a polynucleotide) through methods such as western blot and ELISA. One problem with cell-based bioassays is that they tend to require a large number of cells (*e.g.* 10^3-10^6) and the bioassay results represent the average obtained from a cell population (since homogenates of such a cell population are often used). Under such conditions, the presence of a small percentage of "positive" cells in an assay population may be difficult to detect using conventional devices and methods.

Bioassays have been used extensively in characterizing biological functions of new genes and new biological factors. However, the traditional methods of characterization have been limited to single genes or single biological factors (*i.e.* assaying one gene or one biological factor at a time). Under such conditions, the speed of single gene or single biological factor based functional characterization remains very slow. Under such an approach, the cost would be prohibitively high if applied to characterizing the entire human genome of perhaps 100,000 genes, or to a large population of small molecules or other biological factors. The apparatus and methods described herein which function to localize

positive cells or signals to discrete areas in a highly uniform manner provide a way to assay multiple genes and/or multiple biological factors simultaneously.

With the recent development of high throughput drug screening in the pharmaceutical industry on high density plates, the speed of drug screening has been increased dramatically. However, assay performance on the current screening plates has not been convenient or successful, especially in the case of cell-based drug screening assays performed at high density (*e.g.* plates with density higher than 864 wells). The performance of cell-based assays in this kind of screening environment has been further impeded by the fact that the majority of such assays are of the attached-cell type rather than the suspended-cell type. Limitations in the designs of currently available plates inevitably result in non-homogeneous effects in both the assaying process and in the detection or readout phase. Such non-homogeneous effects are often due to edge phenomena in microliter and sub-microliter fixed-chamber plate designs. For example, when using one type of high density plate (*i.e.* larger than 384 wells), background noise became a serious problem (*see* November 1997, Genetic Engineering News 17, 28; *see also* Owicki et al., November 1997, Genetic Engineering News 17, 27) and confocal microscopy had to be used to detect readout signals. Conventional plate designs will always have the problem of a non-homogeneous cell distribution, contributing to a low signal-to-noise ratio. Conventional plate designs will also always have the problem of an absolute requirement for repetitive dispensing operations, whereas the device of the invention is adaptable to bulk processing (*i.e.* by grid removal). With the plate designs described and set forth herein, these problems can be readily avoided.

3. SUMMARY OF THE INVENTION

This invention relates to designs of an apparatus having a base and a removable grid to create micro-compartments. The apparatus can be used to create local, high concentrations of viruses, microbials, secretable factors and/or other biologics produced from a source of interest for biological screening (*i.e.* functional gene screening, secreted factor screening, *etc.*). The apparatus can also be used in high throughput drug screening procedures, especially in such procedures using cell-based assays, so as to avoid non-homogeneity of assay conditions and to reduce readout noise. This apparatus provides a basis for a massively parallel automated screening process.

In one embodiment, the area of each micro-compartment ranges from 1 μm^2 to 1 cm^2. In another embodiment, the area of each micro-compartment ranges from 100 μm^2 to 100 mm^2. In still another embodiment, the area of each micro-compartment is 1 mm^2.

This invention provides a device for use in screening of a biological assay comprising: (a) a solid support; and (b) a removable grid capable of forming micro-compartments when placed on the solid support. In one embodiment, the removable grid is capable of forming 96 or more micro-compartments when affixed to the solid support. In another embodiment, the removable grid is capable of forming from 384 to 6,144 micro-compartments when affixed to the solid support. In yet another embodiment, the removable grid is capable of forming 1,536 micro-compartments when affixed to the solid support. In yet still another embodiment, the area of each micro-compartment ranges from 1 μm^2 to 1 cm^2. In another embodiment, the area of each micro-compartment ranges from 100 μm^2 to 100 mm^2. In another embodiment, the area of each micro-compartment is 1 mm^2. In another embodiment, the area of the solid support ranges from 1 cm^2 to 10,000 cm^2. In another embodiment, the area of the solid support ranges from 10 cm^2 to 1,000 cm^2. In another embodiment, the area of the solid support is 200 cm^2.

This invention provides a method for screening of a cell-based biological assay comprising: (a) culturing cells on a solid support; (b) contacting cells cultured in step (a) with a population to be screened, the population selected from the group consisting of a cDNA population, a protein population, and a cell population; (c) affixing a removable grid to the solid support to form an assembly; (d) incubating the assembly of the removable grid and the solid support for a suitable period of time; and (e) detecting a biological readout from a biological readout assay, so as to carry out screening of the cell-based biological assay. In one embodiment, the removable grid is capable of forming 96 or more micro-compartments when affixed to the solid support. In another embodiment, the removable grid is capable of forming from 384 to 6,144 micro-compartments when affixed to the solid support. In yet another embodiment, the removable grid is capable of forming 1,536 micro-compartments when affixed to the solid support. In yet still another embodiment, the suitable period of time in step (d) is from about twelve hours to about twelve days. In another embodiment, the suitable period of time in step (d) is from about one day to about three days. In another embodiment, the suitable period of time in step (d) is about two days. In another embodiment, the biological readout assay detects genes in a pathway selected

from the group consisting of a mitogenic signaling pathway, a STAT signaling pathway, an NFκB signaling pathway, a stress signaling pathway, an apoptosis signaling pathway, an NFAT signaling pathway, a Wnt signaling pathway, a CREB signaling pathway, an AP-1 signaling pathway, a proliferation signaling pathway and an anti-proliferation signaling pathway. In another embodiment, detecting the biological readout is carried out using detection means selected from the group consisting of microscopy, immunofluorescence and ELISA.

This invention provides a method for screening of a cell-based biological assay comprising: (a) culturing cells on a solid support; (b) affixing a removable grid to the solid support so as to create discrete micro-compartments; (c) depositing individual samples to be screened into the discrete micro-compartments created in step (b); (d) incubating the assembly of the removable grid and the solid support for a suitable period of time; and (e) detecting a biological readout from a biological readout assay, so as to carry out screening of the cell-based biological assay. In one embodiment, the removable grid is capable of forming 96 or more micro-compartments when affixed to the solid support. In another embodiment, the removable grid is capable of forming from 384 to 6,144 micro-compartments when affixed to the solid support. In yet another embodiment, the removable grid is capable of forming 1,536 micro-compartments when affixed to the solid support. In yet still another embodiment, the suitable period of time in step (d) is from about twelve hours to about twelve days. In another embodiment, the suitable period of time in step (d) is from about one day to about three days. In another embodiment, the suitable period of time in step (d) is about two days. In another embodiment, the biological readout assay detects genes in a pathway selected from the group consisting of a mitogenic signaling pathway, a STAT signaling pathway, an NFκB signaling pathway, a stress signaling pathway, an apoptosis signaling pathway, an NFAT signaling pathway, a Wnt signaling pathway, a CREB signaling pathway, an AP-1 signaling pathway, a proliferation signaling pathway and an anti-proliferation signaling pathway. In another embodiment, detecting the biological readout is carried out using detection means selected from the group consisting of microscopy, immunofluorescence and ELISA. In another embodiment, the individual samples to be screened are selected from the group consisting of oligonucleotides, peptides and small molecules. The above-described methods may be used for drug discovery.

This invention provides a kit for use in screening of a biological assay comprising: (a) a solid support; (b) a removable grid capable of forming micro-compartments when affixed to the solid support; and (c) a description of suggested use. In one embodiment, the solid support is constructed from material selected from the group consisting of glass, plastic, polypropylene, polystyrene and quartz. In another embodiment, the removable grid is constructed from material selected from the group consisting of glass, plastic, polypropylene, polystyrene and quartz. In yet another embodiment, the description of suggested use comprises a description of gene detection in a pathway selected from the group consisting of a mitogenic signaling pathway, a STAT signaling pathway, an NFκB signaling pathway, a stress signaling pathway, an apoptosis signaling pathway, an NFAT signaling pathway, a Wnt signaling pathway, a CREB signaling pathway, an AP-1 signaling pathway, a proliferation signaling pathway and an anti-proliferation signaling pathway.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. A removable wall frame for positioning a micro-compartmentalization device on a cell culture plate.

FIG. 2. The removable wall frame of FIG. 1 attached to a cell culture plate.

FIG. 3. A grid device for carrying out micro-compartmentalization.

FIG. 4. Assembly of a removable wall frame and grid device together on a cell culture plate to create a micro-compartmentalization environment.

5. DETAILED DESCRIPTION OF THE INVENTION

The micro-compartmentalization apparatus is used to create micro-compartmentalized areas or wells on a continuous layer or surface comprising a homogenous population of cells or molecules (*e.g.* receptors, ligands) for the purpose of facilitating high throughput screening, as described in detail below.

5.1. DEVICE

The device of the invention is composed of a base and a removable grid, which grid comprises a desired number of micro-compartments. The base of the micro-compartmentalization apparatus may be any surface (*e.g.* an open tissue culture dish) suitable for culturing cells or depositing substances. Such surface can be in any shape which may be subdivided into micro-compartments. In one embodiment, the surface is designed to be in the same shape as the micro-compartment grid so as to produce a fluid-tight fit when the grid is placed on the surface. The area of each micro-compartment may be virtually any area. In one embodiment, the area of each micro-compartment ranges from 1 μm^2 to 1 cm^2. In another embodiment, the area of each micro-compartment ranges from 100 μm^2 to 100 mm^2. In still another embodiment, the area of each micro-compartment is 1 mm^2. The height of the grid may be virtually any height. In one embodiment, the height of the grid is such that the volume of each micro-compartment ranges from 1 nanoliter to 1 liter. In another embodiment, the height of the grid is such that the volume of each micro-compartment ranges from 1 microliter to 1 milliliter. In another embodiment, the height of the grid is such that the volume of each micro-compartment ranges from 10 microliters to 100 microiliters. In a preferred embodiment, the height of the grid is such that the volume of each micro-compartment is 50 microliters.

A fluid-tight fit between the base and the micro-compartment grid can also be secured by imprinting a grid of the same pattern as the removable grid on the base to form a good seal. Such an imprinted grid on the base may comprise indentations constructed to receive the grid walls. A fluid-tight fit or seal may also be formed by clamping the removable micro-compartment grid onto the base. Here, a fluid-tight seal refers to a seal in which a liquid (*e.g.* a tissue culture medium or an assay buffer) is not permitted to flow between individual micro-compartments.

The micro-compartmentalization apparatus provides a means to convert an assay system effectively from a single sample to multiple samples in a reversible way. That is, the grid can be affixed and removed as desired by the user in order to treat a plate as a single sample or as multiple samples. The reversible nature of the compartmentalization allows a minimum of handling of assay samples since a plate may essentially remain as a single sample during assay setup at the beginning and during assay readout at the end. The conversion from a single sample into a multi-well assay plate prevents the free flow or cross

contamination of biologics (*e.g.* microbials, viruses, polypeptides, small molecules, cells, *etc.*) from one micro-compartment to another, thus achieving a localization effect which may, after a period of time, amplify certain readout signals in particular micro-compartments. For example, where a readout is carried out using retrovirus infection, a high concentration of retrovirus carrying a single recombinant will develop in individual micro-compartments over time. The high concentration permitted to develop in restricted micro-compartments provides for an amplification which facilitates detection. That is, enrichment of positive signals by artificial localization results in a high signal-to-noise ratio which facilitates identifying such positive signals and makes possible the screening of large populations as a single or a few samples.

The usefulness of the device is based, at least in part, on the recognition that the signal-to-noise ratio of a readout assay used to screen a cDNA expression library can be significantly enhanced by localizing multiple molecular copies of each unique clone into discrete regions or compartments. It is the ability to detect a biological readout in heterologous cells which enables the user to identify genes having specific functions. A major advantage of the invention is to provide methods for assaying all genes in a cDNA expression library simultaneously, instead of one-at-a-time, under conditions in which the readout signal-to-noise ratio is significantly enhanced.

In a preferred embodiment, the device is constructed as illustrated in FIG. 1 through FIG. 4. FIG. 1 illustrates a removable wall frame for positioning a micro-compartmentalization grid device on a solid support, such as a cell culture plate. The dimensions of the wall frame can be virtually any dimensions. For example, the length may range from 1 cm to 100 cm, the width may range from 1 cm to 100 cm, and the height may range from 1 mm to 10 cm. In a preferred embodiment, the wall frame is 10 cm long by 15 cm wide by 1 cm in height. FIG. 2 illustrates the removable wall frame of FIG. 1 placed on a solid support, such as a cell culture plate. FIG. 3. illustrates a grid device for carrying out micro-compartmentalization. FIG. 4. illustrates assembly of a removable wall frame and grid device together on a solid support (such as a cell culture plate) to create a micro-compartmentalization environment.

5.2. MATERIALS

The device of the invention may be manufactured from virtually any suitable material. Such suitable materials include but are not limited to glass, plastic, polycarbonate, polyethylene, polypropylene, polystyrene, *etc.*

5.3. AUTOMATION

Automation technology may be applied when using the device and methods of the invention. For example, applying micro-compartmentalization to a readout assay may be automated. Such a readout assay may be as described in detail in copending U.S. Patent Application No. 09/065,775, filed April 24, 1998, entitled "FUNCTION-BASED GENE DISCOVERY," by Cen and Sun (Attorney Docket No. 9557-004), which is incorporated herein by reference in its entirety. Further, the device is suitable for automated immunostaining of the co-culture, and to automated microscopic viewing of the immunostained result.

5.4. FUNCTIONAL SCREENING AND CLONING OF GENES

The following functional screening and cloning approaches for genes may be enhanced using micro-compartmentalization, as described below. Function-based gene discovery is described in detail in copending U.S. Patent Application No. 09/065,775, filed April 24, 1998 (Attorney Docket No. 9557-004), which is incorporated by reference herein in its entirety.

5.4.1. MICRO-COMPARTMENTALIZATION APPLICATIONS FOR GENERATING A SINGLE-TYPE RETROVIRUS-INFECTED CELL POPULATION

The micro-compartmentalization technology of the invention provides a method for enhancement of positive readout signals over background noise when using retroviral vectors as described in detail in copending U.S. Patent Application No. 09/065,775, filed April 24, 1998 (Attorney Docket No. 9557-004). Briefly, the method works by artificially localizing virus particles (and/or cells harboring virus particles) carrying individual clones of a library to a small area. The method may be better understood in the context of the

following considerations. A cDNA library of the human genome is too large (*e.g.* 10^6 clones/library) to be effectively screened simultaneously (*i.e.* as a single pool). To screen a large library effectively, it is preferred that one divide it into from 100 to 1,000 pools, each pool having a diversity of from 10,000 to 1,000 clones, respectively. Using pools of this size, the background noise of a readout assay will preferably be lower than 0.01% to 0.1%, respectively, in order to allow detection of a positive signal. Under these considerations, one can easily see that the ratio of signal-to-noise would be greatly enhanced if a retrovirus vector carrying a positive clone is concentrated into a small area. The micro-compartmentalization technology of the invention provides the means to accomplish this end.

Since a retrovirus-infected cell usually only produces one type of virus, one can artificially create a high concentration of the same virus produced from a single infected producer cell by micro-compartmentalizing producer cells into a small area. When micro-compartmentalization is applied to co-cultures of virus producer cells and readout cells, infection of readout cells with each kind of retrovirus is limited to a small area. In this way, any positive signal generated from a readout cell is restricted, and thereby amplified, in the small area.

In a preferred embodiment, the procedure for micro-compartmentalization may be carried out as follows. A removable wall frame (FIG. 1) may be used to culture readout cells on a flat surface, as illustrated in FIG. 2. A grid apparatus (FIG. 3) may then be placed within the removable wall frame (*e.g.* as in FIG. 4) so as to create a micro-compartmentalization environment for co-culture of readout and producer cells. As previously described above, each micro-compartment restricts infection of the readout cells within it virus carrying one gene. Following co-culture for a suitable time (*e.g.* 1-3 days), the grid and frame may be removed to allow manipulation of the co-culture. The co-culture may be fixed, permeabilized if needed, and immunostained or otherwise treated to detect changes attributable to expressed genes. The co-culture may be read under a microscope or a CCD camera. A cell sample from each positively-staining micro-compartment area is picked out for amplification (*e.g.* by PCR) of the inserted cDNA, subcloning and/or sequencing. If a gene identification attempt fails, positive subpools of cDNA may be selected for further deconvolution (*i.e.* dilution and re-screening) until a single positive clone is obtained. Failed gene identification is indicated by an absence of specific

polymerase chain reaction (PCR) products or by the disappearance of a positive signal when expressing a subcloned PCR product.

A control experiment to ensure acceptable operation of the system may be performed by using a retrovirus encoding the β-galactosidase (β-gal) gene. Proper operation is indicated by demonstrating localized staining of β-gal positive cells by micro-compartmentalization of a co-culture of viral producing cells and target cells.

The readout may be any detectable cellular event, including but not limited to mRNA expression, protein expression, a changed cellular morphology, and a changed localization of a target protein. A change in cellular morphology can be a morphology distinguishable from a parental cell, including cell death (for example apoptosis), cell growth, or cell differentiation. In a preferred embodiment, the readout is a change in reporter gene expression, which reporter gene is under the control of a promoter selected to be activated by genes of interest. In another preferred embodiment, the readout is detected by immunochemistry. Many exemplary readout assays are described in copending U.S. Patent Application No. 09/065,775, filed April 24, 1998 (Attorney Docket No. 9557-004).

5.4.2. MICRO-COMPARTMENTALIZATION APPLICATIONS FOR *IN SITU* ELUTION OF A BEAD-SORTED cDNA LIBRARY FOR TRANSFECTION USING A GENETIC BAR CODE SYSTEM

The micro-compartmentalization device of the invention may be used for in situ elution of a cDNA library which has been sorted onto beads using a genetic bar code system. A "genetic bar code" is an oligonucleotide tag or label having a specific sequence as described in detail in copending U.S. Patent Application No. 09/065,775, filed April 24, 1998 (Attorney Docket No. 9557-004). Briefly, such bar codes provide a method of constructing a cDNA library in a vector containing a plurality of genetic bar codes at a diversity equal to or slightly larger than the diversity of the cDNA library. The micro-compartmentalization device of the invention is useful when beads are used, instead of a gene chip or biological array, for sorting a cDNA library. Here, each individual bead carries multiple copies of one unique complementary bar code. Therefore, each individual bead will hybridize to multiple copies of a single recombinant, thereby sorting and concentrating individual members of the library to discrete loci. As spherical or spheroid supports which

can migrate in solution, beads may provide enhanced hybridization efficiency compared to gene chips or biological arrays. Following hybridization, each bead represents a single, easily manipulable recombinant which may be assayed under a high-throughput pharmaceutical screening format (*e.g.* one bead per well) to determine biological functions of the encoded cDNAs. For example, one can place each unique bead, with a bar-coded vector DNA hybridized thereto, into a well of an assay plate (*e.g.* a 96-well or a 384-well plate). Such placement may be performed robotically. The presence of a low salt solution in each well permits dissociation of vector DNA from the beads. The resulting solution in each well, now containing DNA of a single recombinant, may be mixed with and chemically transfected or electroporated into readout cells. The removable micro-compartmentalization device as described herein may be used during the transfection or electroporation procedure. If such a device is used, then the grid of the device may be removed from the readout cell culture following gene transfer so as to facilitate processing (*i.e.* rinsing, culturing, and assaying for biological function). Any recombinant producing a positive signal in a readout assay may be recovered, for example, by sampling the positive cell population and performing PCR using primers flanking the cDNA insert of the vector.

5.4.3. MICRO-COMPARTMENTALIZATION APPLICATIONS FOR CHEMICAL TRANSFECTION USING BIOLOGICAL ARRAYS OR GENE CHIPS HAVING A FLAT SURFACE

Biological arrays used here are typically constructed on nitrocellulose or nylon. In situ transfection is performed by first immersing a shallow grid for creating micro-compartments in a solution on top of the cells of a tissue culture dish, then contacting a biological array with the hybridized cDNA library on the shallow grid, ensuring that the areas above micro-compartments on the biological array is in full contact with the solution. Such solution may be, for example, standard phosphate buffered saline, tissue culture medium without serum supplement, or any other low-salt solution (solution A). Optimally, each microcompartment corresponds to a unique genetic bar code and therefore one type of recombinant cDNA. The low salt condition of solution A forces dissociation of hybridized cDNA library plasmids from the biological array. Following chemical transfection, cells are rinsed and cultured for a few days (*e.g.* 1-4 days) before performing in situ bioassays.

5.5. SECRETED FACTOR FUNCTIONAL SCREENING

Expression cloning has been quite successful using Cos cells as a cell line for library expression. Cos cells can selectively replicate a particular plasmid of a cDNA library with an SV40 origin into as high as 10^4 copies per cell, thereby ensuring a high expression of a particular cDNA (Munro and Maniatis, 1989, Proc. Natl. Acad. Sci. U.S.A. 86, 9248). Cell surface proteins expressed in a Cos cell system can reach 10^6 molecules per cell (Simmons and Seed, 1988, Nature 333, 568). The abundant expression of a single cDNA in a cell has made possible the expression cloning of several growth factor receptors and membrane-bound ligands by blotting with a specific radiolabeled ligand or receptor (Munro and Maniatis, 1989, Proc. Natl. Acad. Sci. U.S.A. 86, 9248; Davis et al., 1994, Science 266, 816). Considering that secretable growth factors are at least temporarily trapped inside a vesicular compartment of a cell before being exocytosed, a "secretion trap" expression cloning method may be implemented for cloning a secretable factor (*e.g.* angiopoietin-1) by staining the Golgi region of transfected Cos cells with soluble, enzyme-labeled angiopoietin receptors. However, there are no universal function-based expression cloning methods for secretable factors. The device and methods set forth herein will greatly facilitate the discovery of entire families of various growth factors, apoptosis factors, differentiation factors, *etc.*

The invention set forth herein provides for reversible micro-compartmentalization of a homogeneous readout cell population transfected or infected with a cDNA library, where such cell population is cultured as a monolayer in cell culture medium on the base of the micro-compartmentalization apparatus. The implementation of micro-compartmentalization isolates positive signal-producing cells (transfected or infected with a cDNA encoding a secretable factor) from the majority of cells which are non-signal producing, and allows accumulation of a particular signal-generating factor in a high concentration during culture. If a cDNA library is introduced into suitable library-expressing cells (*e.g.* Cos cells) as a mixture, each micro-compartment will likely contain one original factor-producing cell since secretable factors are low abundance genes. If a cDNA library is introduced into library-expressing cells as a sorted library through methods described in copending U.S. Patent Application No. 09/065,775, filed April 24, 1998 (Attorney Docket No. 9557-004), each micro-compartment will contain a population of cells expressing a single cDNA. The concentration of factors in the corresponding micro-

compartment will be much higher in the latter condition. If readout cells are cultured with the library expression cells for a period of time, readout cells will manifest effects induced by a particular factor in certain micro-compartments. For example, if the readout cells are used for detecting growth factor activity, the presence of a high concentration of a growth factor in a micro-compartment will induce readout cells in that compartment to enter S phase and replicate. The bioassays for growth can be, for example, a BRDU incorporation assay. The isolation of the cDNA clone of the particular growth factor can be done by PCR of cDNA inserts from a cDNA-transfected or -infected cell, by sequential deconvolution/siping of the positive pool until a single clone is obtained, or by methods described in the copending application referenced above. The same procedure can be used for cloning apoptosis factors, differentiation factors, *etc.*

5.6. HIGH THROUGHPUT DRUG SCREENING

Many efforts have been made to increase the efficiency of high throughput drug screening through either increasing the speed of drug screening, increasing the well density of each screening plate, or both. Although the well density on a screening plate has increased tremendously (*i.e.* from 96 wells to 384, 864, 1,536, 3,072, 6,144, or even higher), the handling of assay systems and the accuracy of the assay readout has been greatly compromised, especially in cell-based assay systems. For currently-available screening plates, and in conventional practice, it has become very difficult to prepare cells in a uniform manner. One reason is that the volume for dispensing cells into each well is in the microliter or sub-microliter range. Further, the minuscule size and dimensions of each well in the currently available plate designs seems to prevent cells from growing into a homogeneous cell population in each well. As the number of micro-volume pipetting operations increases when using a high well density plate, the variation among individual wells in the assays performed becomes much larger. Both the non-homogeneity of the cell distribution in any individual well and the variation among different wells will ultimately work to lower the signal-to-noise ratio. This is especially true for high density format plate screening. The application of micro-compartmentalization plate designs in high throughput drug screening as set forth in this disclosure solves both of these problems, whether one is using cell-based assay screening or receptor-based assay screening, as described further below.

Because there is a removable grid in the design of the high throughput screening plate set forth herein, one is able to first grow a homogeneous readout cell population on the base of the micro-compartmentalization apparatus using standard tissue culture techniques. One then later positions the micro-compartmentalization grid, containing the desired number of compartment divisions (*e.g.* 96, 384, 864, 1,536, or higher), onto the base so as to form a plate with the desired number of compartments. The homogeneous readout cell population which has been grown on the base ensures that an equal number of cells will be present in each micro-compartment once the grid is affixed. This procedure ensures that there will be no adverse effects attributable to non-homogeneous cell distribution, thus overcoming a serious problem encountered when using conventional screening plates. A population to be screened for bioactivity may be contacted with the homogeneous readout cell population grown on the base of the plate either before or after affixing the grid device to effect micro-compartmentalization. Such populations to be screened for bioactivity may include a small molecule population, a protein population, a cDNA population, a cell line comprising a cDNA population, or any other population that may contain bioactivity known to one skilled in the art. After incubating the population to be screened for a suitable period of time after affixing the grid (determined by the specific assay being performed), one can remove the micro-compartmentalization grid such that the base plate may be treated or processed as a single sample for *in situ* bioassays, such as *in situ* biochemistry assays and *in situ* cell biology assays. Alternatively, one may keep the micro-compartmentalization grid in place during a readout assay. However, the removal of the grid and treatment of an entire plate as a single sample not only avoids thousands of micro-volume pipetting operations, but also avoids well-to-well variation during processing. The results of *in situ* bioassays may be read, for example, under a microscope or other device capable of distinguishing among small, discrete micro-compartment areas. The identification of a positive population of readout cells indicates a "hit" for whatever small molecule or other biologic corresponds to the specific micro-compartment where the positive signal is located.

Additional quantitative parameters, such as K_i and IC_{50}, may be determined in a subsequent analysis. Alternatively, such quantitative parameters may also be measured, to a first approximation, using the intensity of the positive cell readout. This is possible because of the high signal-to-noise ratio that can be achieved under the homogeneous conditions provided by the device and methods of the invention. Virtually any assay system will yield

some quantitative information when used together with the micro-compartmentalization drug-screening plates of the invention. Such information is not usually obtainable when using conventional screening plates because of the low signal-to-noise ratio attributable to variation in the assays from well-to-well.

In case of a cell suspension assay system, cells are first prepared as a homogenous layer on the base of the micro-compartment device through, for example, binding to antibodies which recognize cell surface antigens. In this instance, such antibodies are pre-coated onto the base of the micro-compartment device. The micro-compartmentalization grid can then be applied as described above. The final readout can be measured with or without removal of the micro-compartment grid.

The same procedure can also be applied to binding-based assay screening, so long as an *in situ* bioassay is available to, or can be designed to, detect the result of binding. Many such binding-based assays are well known to one skilled in the art and are commonly used in the pharmaceutical industry. For example, in using a receptor-ligand binding assay, one would first coat the base of the micro-compartment apparatus with a molecule (*e.g.* the receptor), or homogeneous population of molecules, to be screened for binding activity. Next the micro-compartmentalization grid is attached to the base so as to convert it into a multi-well screening plate (*e.g.* 96, 384, 864, 1,536, 3,072, 6,144, or more wells). A different test agent (*e.g.* chemicals, compounds, oligonucleotides, peptides, peptido-mimetics, small molecules, *etc.*) may then be added into each distinct micro-compartments and incubated for a suitable period of time to allow any binding to occur. Such suitable time periods are well known to one skilled in the art for various binding assays. In one detection scheme, the incubation period is followed by removal of the micro-compartment grid and addition of a solution containing a ligand for the receptor. Antibodies recognizing the ligand, followed by enzyme-linked second antibodies, may be used for *in situ* detection of receptor-ligand complexes. In this example, the failure to detect ligand molecules in a particular micro-compartment indicates a "hit" by the particular test agent incubated in that micro-compartment.

A number of specific exemplary biological readout assays which may be used in conjunction with the device and methods of the invention are set forth in detail below.

5.7. CELLULAR READOUT ASSAYS

5.7.1. PROLIFERATION PATHWAY

Bromodeoxyuridine (BRDU) incorporation may be used as an assay to identify genes involved in proliferation. The BRDU assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (*see* Hoshino *et al.*, 1986, Int. J. Cancer 38, 369; Campana *et al.*, 1988, J. Immunol. Meth. 107, 79).

A proliferating cell nuclear antigen (PCNA) assay may also be used to identify genes involved in cell proliferation. PCNA (*a.k.a.* cyclin or the polymerase d associated protein) is a 36 kilodalton protein whose expression is elevated in proliferating cells. PCNA is synthesized in early G1 and S phases of the cell cycle and therefore serves as an excellent marker for proliferating cells. Positive cells are identified by immunostaining using an anti-PCNA antibody (*see* Li *et al.*, 1996, Current Biology 6, 189; Vassilev *et al.*, 1995, J. Cell Sci. 108, 1205).

5.7.2. STRESS SIGNALING PATHWAY

p53 is an important modulator of the stress response. p53-dependent transcriptional activation may therefore be used to identify genes involved in a stress signaling pathway. A readout cell population containing a reporter gene under the control of a p53-inducible promoter may be used for the assay. Suitable reporter genes include, but are not limited to, β-galactosidase (β-gal), chloramphenicol acetyltransferase (CAT), and luciferase. Positive cells may be identified by blue color in a β-gal reporter gene assay (*see e.g.* Komarova *et al.*, 1997, EMBO J. 16, 1391-1400) or by immunostaining for the reporter gene product. A p53 induction assay may also be used to identify genes involved in a stress signaling pathway. p53 induction (*i.e.* increases in cellular p53 protein expression) may be identified by immunostaining using a specific anti-p53 antibody (Anker *et al.*, 1993, Int. J. Cancer 55, 982; Weiss *et al.*, 1993, Int. J. Cancer 54, 693).

A heat shock transcription factor 3 (HSF3) aggregation assay may also be used to identify genes in a stress signaling pathway. The HSF3 aggregation assay measures HSF3 aggregation in the nucleus induced by cellular stress signals through immunostaining using a specific anti-HSF3 antibody (Kanei-Ishii *et al.*, 1997, Science 277, 246).

An activated c-Jun kinase assay may be used to identify genes in a stress signaling pathway. c-Jun kinase (JNK) is a transcription factor which is activated by phosphorylation (p-JNK). Many stress signals result in activation of c-Jun kinase by phosphorylation (Derijard *et al.*, 1994, Cell 76, 1025). The availability of p-JNK specific antibodies (Santa Cruz) allows *in situ* detection of cells in which JNK is activated by heterologous library genes.

5.7.3. LOSS OF INVASIVENESS

Invasion inhibition assays may be used to identify genes involved in cancer. One such assay measures induction of E-cadherin-mediated cell-cell adhesion. The induction of E-cadherin-mediated adhesion can result in phenotypic reversion and loss of invasiveness of epithelial cells. This assay measures increased expression of E-cadherin at the cell junction through immunostaining using a specific anti-E-cadherin antibody (Hordijk *et al.*, 1997, Science 278, 1464). Another such assay measures loss of hepatocyte growth factor (HGF)-induced cell scattering. Loss of HGF-induced cell scattering is correlated with loss of invasiveness of epithelial cells such as Madin-Darby canine kidney (MDCK) cells. This assay identifies a cell population which has lost cell scattering activity in response to HGF and therefore forms compact colonies (Hordijk *et al.*, 1997, Science 278, 1464).

5.7.4. APOPTOSIS SIGNALING PATHWAY

One assay for apoptosis is the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation, the hallmark of apoptosis in many cell types (*see e.g.* Lazebnik *et al.*, 1994, Nature 371, 346), by following the incorporation of fluorescein-dUTP (Yonehara *et al.*, 1989, J. Exp. Med. 169, 1747). These assay kits are commercially available through suppliers such as Clontech and Boehringer Mannheim.

5.7.5. ANTI-PROLIFERATION PATHWAY

One assay useful for gene identification in an anti-proliferation signaling pathway is the p15 induction assay. p15 is a member of a family of specific inhibitors of Cdk4 and Cdk6. The latter are essential for G1 progression into S phase of the cell cycle (Sherr *et al.*, 1995, Genes & Dev. 9, 1149). The expression of p15 is positively regulated by transforming growth factor-β (Reynisdottir *et al.*, 1997, Genes & Dev. 11, 492). p15 induction may be identified by immunostaining using a specific anti-p15 antibody available commercially (*e.g.* Santa Cruz).

Another assay useful for gene identification in an anti-proliferation signaling pathway is the p21 induction assay. Increased levels of p21 expression in cells results in Cdk inhibition, thus resulting in delayed entry into G1 of the cell cycle (Harper *et al.*, 1993, Cell 75, 805; Li *et al.*, 1996, Current Biology 6, 189). For example, p21 expression can be elevated by p53 and transforming growth factor-β activities. p21 induction may be identified by immunostaining using a specific anti-p21 antibody available commercially (*e.g.* Santa Cruz).

Yet another assay useful for gene identification in an anti-proliferation signaling pathway is the p27 induction assay. As for the assays above, p27 is also a member of the Cdk inhibitor family of proteins. The expression of p27 is increased upon mitogen withdrawal or contact inhibition (Polyak *et al.*, 1994, Cell 78, 59). p27 induction may be identified by immunostaining using a specific anti-p27 antibody available commercially (*e.g.* Santa Cruz).

5.7.6. WNT SIGNALING PATHWAY

One assay for detection of genes which modulate the Wnt signaling pathway is a β-catenin induction and/or translocation assay. The activation of the Wnt signaling pathway results in an increased expression of β-catenin and the translocation of β-catenin from the cytoplasmic compartment to the nucleus (Kuhl *et al.*, 1997, BioEssays 19, 101). This assay is used to identify cells and/or cell populations in which the expression of β-catenin is increased compared to background levels, and/or in which a change of β-catenin localization occurs, in response to expression of a heterologous gene. Changes in

β-catenin expression or localization are detected using a specific anti-β-catenin antibody (*e.g.* Tao *et al.*, 1996, J. Cell Biol. 134, 1271).

Another assay for detection of genes which modulate the Wnt signaling pathway is a LEF-1 inducible promoter induction assay. β-catenin activates downstream targets in the Wnt signaling pathway by binding to a transcription factor known as LEF-1, thus resulting in activation of a LEF-1 inducible promoter (Korinek *et al.*, 1997, Science 275, 1785). A readout cell line containing a reporter gene, such as β-gal, under a LEF-1 inducible promoter is used for the assay. When β-gal is used as a reporter gene, positive cells are the darker blue cells.

5.7.7. STAT SIGNALING PATHWAY

The STAT (signal transducers and activators of transcription) signaling pathway is activated by many growth factors and cytokines and plays essential roles in cell differentiation, cell cycle control, and development. There are six known members of the STAT transcription factor family. Each STAT family member (except STAT2) is known to recognize a specific DNA binding sequence (Ihle, 1996, Cell 84, 331). The assay employs a readout cell line containing a reporter gene, such as β-gal, under the control of any of these known STAT-inducible promoters (White *et al.*, 1996, Cytokine Growth Factor Rev. 7, 303). Positive cells stain dark blue when β-gal is used as the reporter gene. This assay may be used to identify genes in a STAT1 signaling pathway, a STAT3 signaling pathway, a STAT4 signaling pathway, and/or a STAT5/STAT6 signaling pathway. Since STAT5 and STAT6 share the same DNA recognition site, the assay does not distinguish between these two STAT pathways. Readout cells expressing a gene which activates a particular STAT transcription factor will produce a positive signal. Accordingly, the genes identified reside just upstream in the particular STAT pathway assayed.

5.7.8. MAP KINASE SIGNALING PATHWAY

MAP kinase signaling pathway genes may be identified using a p-ERK assay. The activation of this signal transduction pathway by certain growth factors, hormones and neurotransmitters is mediated through two closely-related MAP kinases, p44 and p42, also known as ERK1 and ERK2. ERK proteins are activated by dual phosphorylation at specific tyrosine and threonine sites. The p-ERK assay is used to identify genes by immunostaining

readout cells with an antibody which specifically detects the presence of phosphorylated ERK (p-ERK). Such p-ERK antibodies, which only recognize phosphorylated ERK1 and ERK2, may be obtained commercially (*e.g.* Santa Cruz). *See* Boulton *et al.*, 1991, Cell 65, 663.

5.7.9. AP-1 SIGNALING PATHWAY

Genes in an AP-1 signaling pathway may be identified using a c-fos induction readout assay. The AP-1 signaling pathway is involved in cell proliferation, cell survival and cell stress. Activation of the AP-1 signaling pathway results in an increased expression of genes under the control of an AP-1 promoter sequence such as the c-fos gene (*see e.g.* Karin *et al.*, 1997, Curr. Opin. Cell Biol. 9, 240). The c-fos induction assay identifies genes expressed in cell populations in which the level of endogenous c-fos protein is increased by immunostaining c-fos using a specific anti-c-fos antibody (Telford *et al.*, 1996, J. Comp. Neurol. 375, 601).

5.7.10. CREB SIGNALING PATHWAY

In one embodiment, genes in a cyclic adenosine monophosphate response element binding protein (CREB) signaling pathway may be identified using a phosphorylated CREB (p-CREB) readout assay. CREB is activated by phosphorylation following an increase in the intracellular concentration of cAMP or Ca^{2+}. An antibody which specifically recognizes phosphorylated CREB allows detection of an activated CREB pathway in readout cells (Ginty *et al.*, 1994, Cell 77, 713).

In another embodiment, genes in a CREB signaling pathway may be identified using a cyclic adenosine monophosphate response element (CRE) reporter gene assay. In this assay, a readout cell containing a reporter gene (*e.g.* β-gal, CAT or luciferase) under the control of the CRE is used for the assay. Positive cells may be identified by, *e.g.*, blue staining in a β-gal assay (Himmler *et al.*, 1993, J. Recept. Res. 13, 79; Kruger *et al.*, 1997, Naunyn Schmiedebergs Arch. Pharmacol. 356, 433) or by immunostaining for the reporter gene product.

5.7.11. NFκB SIGNALING PATHWAY

In one embodiment, an NFκB translocation assay may be used to identify genes in an NFκB signaling pathway. Activation of the NFκB signaling pathway results in translocation of NFκB from the cytoplasm to the nucleus. The NFκB translocation assay identifies cells with NFκB translocated to the nucleus by immunostaining for NFκB using a specific anti-NFκB antibody (Han *et al.*, 1997, J. Biol. Chem. 272, 9825; Janssen *et al.*, 1995, Adv. Cancer Res. 151, 389).

In another embodiment, an NFκB reporter gene assay may be used to identify genes in an NFκB signaling pathway. In this assay, a readout cell containing a reporter gene (*e.g.* β-gal, CAT or luciferase) under the control of an NFκB response element is used for the assay. Positive cells may be identified, *e.g.*, by blue staining in a β-gal assay or by immunostaining for the reporter gene product (Rothe *et al.*, 1995, Science 269, 1424).

5.7.12. NFAT SIGNALING PATHWAY

Genes in an NFAT signaling pathway may be identified using a NFAT reporter gene assay. In this assay, a readout cell expressing a reporter gene (*e.g.* β-gal, CAT or luciferase) under the control of an NFAT response element is used. Positive clones may be identified by blue staining in a β-gal assay (*see e.g.* Burres *et al.*, 1995, J. Antibiot. 48, 380) or by immunostaining for the reporter gene product.

5.7.13. INSULIN SIGNALING PATHWAY

Genes in the insulin signaling pathway may be identified using a GLU4 translocation assay. Insulin stimulation of adipocytes results in translocation of the GLU4 glucose transporter to the plasma membrane. This assay identifies cells in which the insulin signaling pathway is activated by immunostaining GLU4 protein localized at the plasma membrane (Martin *et al.*, 1996, J. Biol. Chem. 271, 17605).

5.7.14. MDR SIGNALING PATHWAY

Genes in the multiple drug resistance (MDR) gene regulation pathway may be identified using an MDR reporter gene assay. MDR gene expression is often greatly increased in cancer cells resistant to chemotherapy. In this assay, a readout cell containing a reporter gene (*e.g.* β-gal, CAT or luciferase) under the control of an MDR gene promoter may be used for the assay. Positive cells may be identified by blue staining in a β-gal assay (Walther *et al.*, 1997, Gene Ther. 4, 544) or by immunostaining using an antibody specific for the reporter gene product.

5.7.15. CHOLESTEROL TRANSPORT PATHWAY

Genes important in a cholesterol transport pathway may be identified using an intracellular cholesterol accumulation assay. For example, mutations of the Niemann-Pick type C (NP-C) gene result in lysosomal accumulation of low density lipoprotein (LDL)-derived cholesterol. The accumulated cholesterol in the cytoplasm is detected by staining with filipin, a specific cytochemical marker of unesterified cholesterol. The filipin staining assay may be used to identify cells with cholesterol accumulation due to the expression an exogenous sense or anti-sense cDNA (*see* Eugene *et al.*, 1997, Science 277, 228).

5.8. BIOCHEMICAL READOUT ASSAYS

In the practice of this invention, biochemical readout assays may be used to identify genes modifying specific activities following *in vitro* transcription and translation. Such biochemical readout assays include, but are not limited to, enzymatic and receptor-based assays. There are a wide variety of assays for enzymatic activities and receptor-binding activities which may be adapted for use in identification of new genes upon screening a library of interest, as further exemplified in this Section below.

There are many resources available describing such enzymatic and receptor-based assays suitable for use with the methods of the invention. For example, *Methods in Enzymology* is a multi-volume reference published by Academic Press which describes biological methods, including enzymatic and receptor-based assays, in detail. Further, Fernandez-Botran and Vetvicka, 1995, *Methods in Cellular Immunology*, CRC Press, describes assays for immune cell activation, including cytokine receptor assays.

Biochemical readout assays may include, *e.g.*, detection of: GABA receptor activity, glutamate receptor activity, monoamine oxidase activity, nitric oxide synthetase activity, opiate receptor activity, serotonin receptor activity, adenosine A_1 agonist and antagonist activity, adrenergic α_1, α_2, β_1 agonist and antagonist activity, calcium channel blocker activity, inflammatory mediator activity, such as the interleukins (*e.g.* IL-1, IL-6), tumor necrosis factor activity, arachidonic acid activity and phosphatase activity (*e.g.* tyrosine phosphatase). Further, biochemical readout assays may include binding to protein domain or subdomain, for example, a PDZ domain, a PH domain, an SH2 domain, and an SH3 domain. Still further, biochemical readout assays may include binding to a molecule, for example, phosphotyrosine and phosphorylated inositol. A functional assignment given to a particular gene may be derived from results obtained in more than one assay. Indeed, it is preferred that a functional assignment be derived from results obtained in a panel of two or more assays. Generally, one skilled in the art would know which assays are appropriate to employ to best identify genes having, or modifying, a particular function-of-interest.

Further specific examples of assays based on enzymes or receptors include the following: acetylcholinesterase; aldol-reductase; angiotensin converting enzyme (ACE); cyclooxygenases; DNA repair; β-glucuronidase; lipoxygenases; monoamine oxidases; phospholipase A_2, platelet activating factor (PAF); potassium channel assays; prostaglandin synthetase; serotonin re-uptake activity; and steroid receptors. Additional assays may include: ATPase inhibition, benzopyrene hydroxylase inhibition, HMG-CoA reductase inhibition, phosphodiesterase inhibition, protease inhibition, and tyrosine kinase inhibition.

5.9. USER-DEFINED ASSAYS

The methods of the invention are not limited to use with the readout assays described herein. Such readout assays merely serve to exemplify a few of the myriad possibilities suitable for use with the invention. When the readout assay is a cellular readout assay, virtually any cell line identified as suitable by one skilled in the art may be used. Further, virtually any reporter gene, or endogenous gene functioning as a reporter gene, identified as suitable by one skilled in the art may be used. It will be well noted by one skilled in the art that the methods of the invention are suitable for use with any known readout assay, whether the assay be cellular or biochemical.

The skilled practitioner will recognize that it is the particular readout assay, whether chosen from the literature or designed by the user, which determines the type (*i.e.* function) of genes identified. For example, if one wishes to identify genes associated with cancer, one may choose to screen the library of interest using the p53 and or MDR assays described above. Often, the user will provide the most appropriate readout assay to be employed for identification of particular genes-of-interest.

5.10. SCREENING DIVERSITY LIBRARIES

In one embodiment, diversity libraries and/or chemical libraries may be screened using the device and methods of the invention. Many such libraries are commercially available (*e.g.*, libraries are available from ArQule, Tripos/PanLabs, ChemDesign, and Pharmacopoeia). Any such library known in the art can be used to provide molecules to be screened according to the present invention. Alternatively, such libraries can be constructed using standard methods. Chemical (synthetic) libraries, recombinant expression libraries, or polysome-based libraries are exemplary types of libraries that can be used.

A diversity library can be a cDNA or genomic expression library, a random peptide expression library or a chemically-synthesized random peptide library or synthetic organic compound library, *etc.* In one embodiment, the peptide libraries used in the present invention may be libraries that are chemically synthesized *in vitro*. Examples of such libraries are given by Houghten et al. (1991, Nature 354, 84-86), who describe mixtures of free hexapeptides in which the first and second residues in each peptide are individually and specifically defined; Lam et al. (1991, Nature 354, 82-84), who describes a "one bead, one peptide" approach in which a solid phase split synthesis scheme produced a library of peptides in which each bead in the collection had immobilized thereon a single, random sequence of amino acid residues; Medynski (1994, Bio/Technology 12, 709-710) who describes split synthesis and T-bag synthesis methods; and Gallop et al. (1994, J. Medicinal Chemistry 37, 1233-1251). A combinatorial library may be prepared for use, *e.g.*, according to the methods of Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. U.S.A. 90, 10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91, 11422-11426; Houghten et al., 1992, Biotechniques 13, 412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91, 1614-1618; or Salmon et al., 1993, Proc. Natl. Acad. Sci. U.S.A. 90, 11708-11712. PCT Publication No. WO 93/20242 and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci.

U.S.A. 89, 5381-5383 describe "encoded combinatorial chemical libraries," that contain oligonucleotide identifiers for each chemical polymer library member. Compounds synthesized so as to be immobilized on a substrate are released from the substrate prior to use in the inhibition assay.

Further, more general, structurally constrained, organic diversity (*e.g.*, non-peptide) libraries, can also be used. By way of example, a benzodiazepine library (*see e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91, 4708-4712) may be used.

Conformationally-constrained libraries that can be used include but are not limited to those containing invariant cysteine residues which, in an oxidizing environment, cross-link by disulfide bonds to form cystines, modified peptides (*e.g.*, incorporating fluorine, metals, isotopic labels, are phosphorylated, *etc.*), peptides containing one or more non-naturally occurring amino acids, non-peptide structures, and peptides containing a significant fraction of γ-carboxyglutamic acid.

Libraries of non-peptides, *e.g.*, peptide derivatives (for example, that contain one or more non-naturally occurring amino acids) can also be used. One example of these are peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89, 9367-9371). Peptoids are polymers of non-natural amino acids that have naturally occurring side chains attached not to the alpha carbon but to the backbone amino nitrogen. Since peptoids are not easily degraded by human digestive enzymes, they are advantageously more easily adaptable to drug use. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91, 11138-11142).

The members of the libraries that can be screened according to the invention are not limited to containing the 20 naturally occurring amino acids. In particular, chemically-synthesized libraries and polysome-based libraries allow the use of amino acids in addition to the 20 naturally occurring amino acids (by their inclusion in the precursor pool of amino acids used in library production). In specific embodiments, the library members contain one or more non-natural or non-classical amino acids or cyclic peptides. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid; γ-Abu, ϵ-Ahx, 6-amino hexanoic acid; Aib, 2-amino isobutyric acid; 3-amino propionic acid; ornithine;

norleucine; norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, ß-alanine, designer amino acids such as ß-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, fluoro-amino acids and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed since these embodiments are intended as illustration of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. Throughout this application various publications, patents, and patent applications are cited. Their contents are hereby incorporated by reference into the present application in their entireties.

We claim:

1. A device for use in screening of a biological assay comprising:
   (a) a solid support; and
   (b) a removable grid capable of forming micro-compartments when placed on the solid support.

2. The device of Claim 1, wherein the removable grid is capable of forming 96 or more micro-compartments when affixed to the solid support.

3. The device of Claim 1, wherein the removable grid is capable of forming from 384 to 6,144 micro-compartments when affixed to the solid support.

4. The device of Claim 1, wherein the removable grid is capable of forming 1,536 micro-compartments when affixed to the solid support.

5. The device of Claim 1, wherein the area of each micro-compartment ranges from 1 μm^2 to 10 mm^2.

6. The device of Claim 1, wherein the area of each micro-compartment ranges from 100 μm^2 to 2 mm^2.

7. The device of Claim 1, wherein the area of each micro-compartment is 1 mm^2.

8. The device of Claim 1, wherein the area of the solid support ranges from 1 cm^2 to 10,000 cm^2.

9. The device of Claim 1, wherein the area of the solid support ranges from 10 cm^2 to 1,000 cm^2.

10. The device of Claim 1, wherein the area of the solid support is 200 cm^2.

11. A method for screening a population using a cell-based biological readout assay comprising the following steps in the order stated:

(a) contacting cells cultured on a surface of a solid support with a population to be screened, the population selected from the group consisting of a molecule population and a cell population;

(b) affixing a removable grid to the surface of the solid support containing the contacted cells;

(c) incubating the cells for a period of time suitable to the biological readout assay; and

(d) detecting a biological readout from the biological readout assay,

so as to carry out screening of the population using the biological readout assay.

12. The method of Claim 11, wherein the removable grid is capable of forming 96 or more micro-compartments when affixed to the surface of the solid support.

13. The method of Claim 11, wherein the removable grid is capable of forming from 384 to 6,144 micro-compartments when affixed to the surface of the solid support.

14. The method of Claim 11, wherein the removable grid is capable of forming 1,536 micro-compartments when affixed to the surface of the solid support.

15. The method of Claim 11, wherein the suitable period of time in step (c) is in the range of twelve hours to twelve days.

16. The method of Claim 11, wherein the suitable period of time in step (c) is in the range of one day to three days.

17. The method of Claim 11, wherein the suitable period of time in step (c) is two days.

18. The method of Claim 11, wherein the biological readout assay detects genes in a pathway selected from the group consisting of a mitogenic signaling pathway, a STAT

signaling pathway, an NFκB signaling pathway, a stress signaling pathway, an apoptosis signaling pathway, an NFAT signaling pathway, a Wnt signaling pathway, a CREB signaling pathway, an AP-1 signaling pathway, a proliferation signaling pathway and an anti-proliferation signaling pathway.

19. The method of Claim 11, wherein detecting the biological readout is carried out using detection means selected from the group consisting of immunofluorescence and ELISA.

20. A method for screening of a population using a cell-based biological assay comprising:

(a) culturing cells on a surface of a solid support;

(b) affixing a removable grid to the surface of the solid support so as to create discrete micro-compartments;

(c) depositing individual samples to be screened into the discrete micro-compartments created in step (b);

(d) incubating the samples in the micro-compartments for a suitable period of time; and

(e) detecting a biological readout from a biological readout assay

so as to carry out screening of the population using the cell-based biological assay.

21. The method of Claim 20, wherein the removable grid is capable of forming 96 or more micro-compartments when affixed to the surface of the solid support.

22. The method of Claim 20, wherein the removable grid is capable of forming from 384 to 6,144 micro-compartments when affixed to the surface of the solid support.

23. The method of Claim 20, wherein the removable grid is capable of forming 1,536 micro-compartments when affixed to the surface of the solid support.

24. The method of Claim 20, wherein the suitable period of time in step (d) is in the range of twelve hours to twelve days.

25. The method of Claim 20, wherein the suitable period of time in step (d) is in the range of one day to three days.

26. The method of Claim 20, wherein the suitable period of time in step (d) is two days.

27. The method of Claim 20, wherein the biological readout assay detects genes in a pathway selected from the group consisting of a mitogenic signaling pathway, a STAT signaling pathway, an NFκB signaling pathway, a stress signaling pathway, an apoptosis signaling pathway, an NFAT signaling pathway, a Wnt signaling pathway, a CREB signaling pathway, an AP-1 signaling pathway, a proliferation signaling pathway and an anti-proliferation signaling pathway.

28. The method of Claim 20, wherein detecting the biological readout is carried out using detection means selected from the group consisting of immunofluorescence and ELISA.

29. The method of Claim 20, wherein the individual samples to be screened are selected from the group consisting of oligonucleotides, peptides and small molecules.

30. A kit for use in screening of a biological assay comprising:

(a) a packaged sterile solid support;

(b) a removable grid capable of forming micro-compartments when affixed to the solid support; and

(c) a description of suggested use.

31. The kit of Claim 30, wherein the solid support is constructed from material selected from the group consisting of glass, plastic, polypropylene, polystyrene and quartz.

32. The kit of Claim 30, wherein the removable grid is constructed from material selected from the group consisting of glass, plastic, polypropylene, polystyrene and quartz.

33. The kit of Claim 30, wherein the description of suggested use comprises a description of gene detection in a pathway selected from the group consisting of a mitogenic signaling pathway, a STAT signaling pathway, an NFκB signaling pathway, a stress signaling pathway, an apoptosis signaling pathway, an NFAT signaling pathway, a Wnt signaling pathway, a CREB signaling pathway, an AP-1 signaling pathway, a proliferation signaling pathway and an anti-proliferation signaling pathway.

34. The method of Claim 11, wherein the period of time suitable to the biological readout assay is in the range of 12 hours to 12 days.

35. The method of Claim 11, wherein the period of time suitable to the biological readout assay is in the range of 1 day to 6 days.

36. The method of Claim 11, wherein the period of time suitable to the biological readout assay is in the range of 2 days to 3 days.

37. A device for use in screening of a biological assay comprising:

(a) a solid support;

(b) a removable grid capable of forming micro-compartments when placed on the solid support; and

(c) a removable wall frame for positioning the removable grid on the solid support.

38. The device of claim 37, wherein the solid support is a cell culture plate.

39. The device of claim 37, wherein the dimensions of the removable wall frame are 10 cm long by 15 cm wide by 1 cm high.

40. The method of claim 11, wherein the molecule population is a diversity library.

41. The device of claim 1, further comprising cells situated on the solid support.

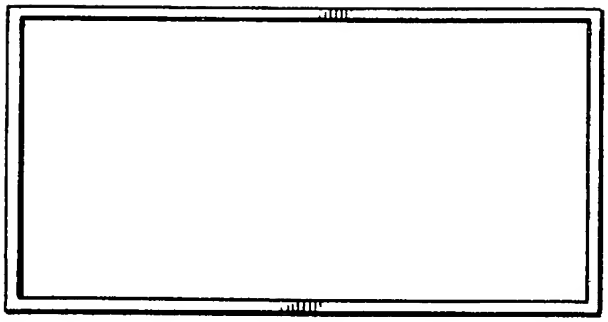


FIG. 1A

FIG. 1B

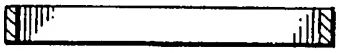


FIG. 1C

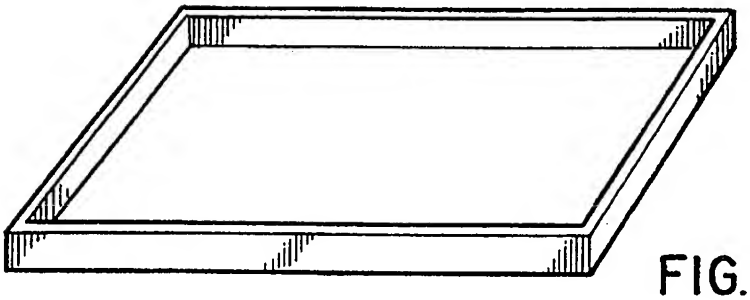


FIG. 1D

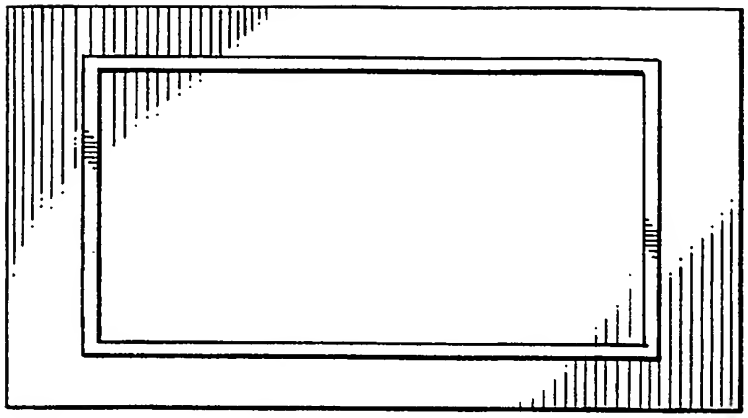


FIG. 2A

FIG. 2B

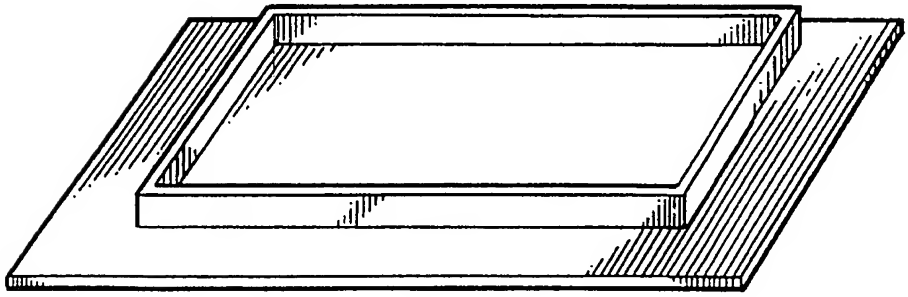


FIG. 2C

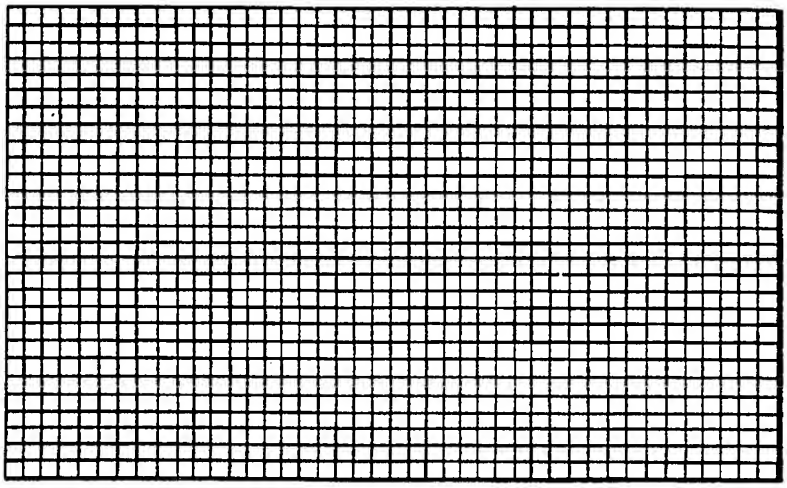


FIG. 3A

FIG. 3B

FIG. 3C

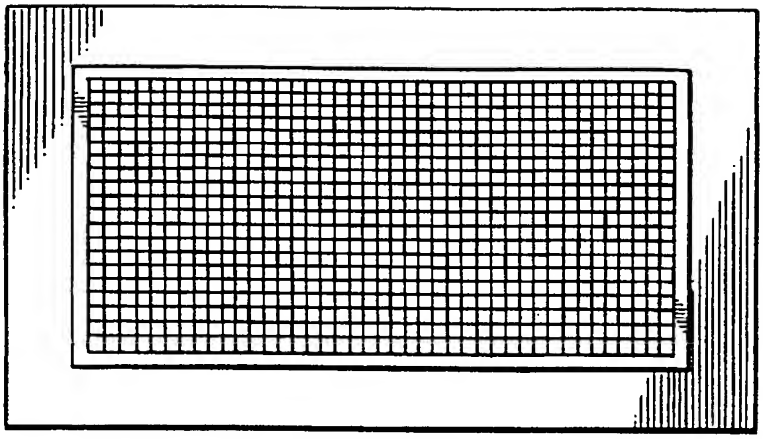


FIG. 4A

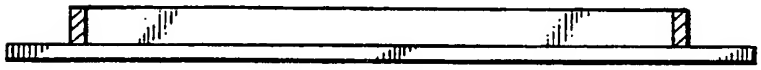


FIG. 4B

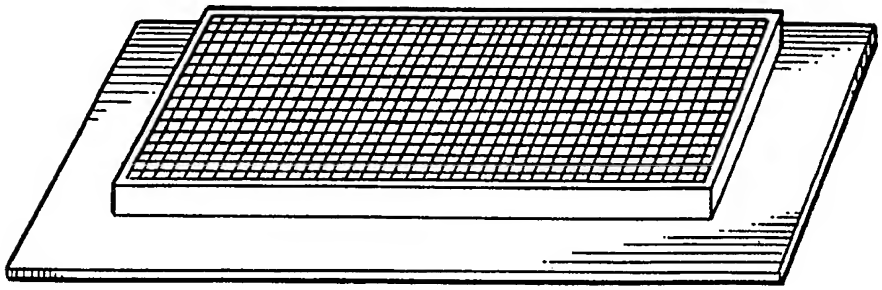


FIG. 4C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08684

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12M 1/18
US CL :435/288.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/288.4, 288.7, 305.2, 305.3; 422/102; 356/246; 359/398; 220/529

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 4,299,920 A (PETERS) 10 November 1981 (10.11.81), see entire document.	1, 2, 5, 6, 8, 9, 41 ---------- 3, 4, 7, 10, 30-33, 37-39
X ---- A	WO 91/09970 A1 (WILLINGHAM ET AL.) 11 July 1991 (11.07.91), see entire document.	1, 8, 41 ---------- 2-7, 9, 10, 30-33, 37-39
X ---- A	US 3,745,091 A (MCCORMICK) 10 July 1973 (10.07.73), see entire document.	1, 41 ---------- 2-10, 30-33, 37-39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
12 AUGUST 1999	25 AUG 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer William H. Beisner Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet)(July 1992)☆

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08684

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 3,656,833 A (WALLACE) 18 April 1972 (18.04.72), see entire document.	1, 37, 38, 41 ---------- 2-10, 30-33, 39
X ---- A	US 4,039,247 A (LAWMAN ET AL.) 02 August 1977 (02.08.77), see entire document.	1 ---------- 2-10, 30-33, 37-39, 41
A	SU 1477739 A1 (LVOV MED INST) 07 May 1989 (07.05.89), see entire document.	37-39
Y	US 4,011,350 A (MARKOVITS ET AL.) 08 March 1977 (08.03.77), see entire document.	30-33
Y, P	US 5,858,309 A (MATHUS ET AL.) 12 January 1999 (12.01.99), see entire document.	3-4, 33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08684

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-10, 30-33, 37-39 and 41

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/08684

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

I. Claims 1-10, 30-33, 37-39 and 41 drawn to device and kit.
II. Claims 11-29, 34-36 and 40 drawn to method of screening.

Furthermore, a lack of unity with respect to the species of the following claims as follows:

Claims 18, 27, 33 are deemed to correspond to the species listed in the following manner:
Group I: a). mitogenic signaling pathway,
Group II: b). STAT signaling pathway,
Group III: c). Nfk B signaling pathway,
Group IV: d). stress signaling pathway,
Group V: e). apoptosis signaling pathway,
Group VI: f). NFAT signaling pathway,
Group VII: g). Wnt signaling pathway,
Group VIII: h). CREb signaling pathway,
Group IX i). AP-1 signaling pathway,
Group X: j). proliferation signaling pathway,
Group XI: k). anti-proliferation signaling pathway.

The following claims are generic: 11, 20, 30

The inventions listed as Groups I and II do not relate to a single invention concept under PCT Rule 13.1 because, under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons: the device lack the special technical features of a device as disclosed by the apparatus of Ramsay (Nature Biotechnology) or the process as claimed lack the special or technical features as for functional gene screening or secreted factor screening to create local high concentrations of viruses, microbes, secretable factors and/or other biologics produced from a source of interest using device with peripheral skirts or for automated immunostaining of the co-culture and to automated microscopic viewing of the immunostained result. Therefore, the inventions of Groups I and II do not share a special technical feature within the meaning of PCT Rule 132.1 so as to form a single general inventive concept.

Each of the recited species has require different operating conditions or materials or different means of identifying or

assaying for a gene that would require different considerations. For example, the proliferation pathway may incorporate compounds such as bromodeoxyuridine to identify genes involved in proliferation while in stress signaling pathway a modulator as p53 may be used to identify genes involved in a stress signaling pathway. An assay for apoptosis is the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling assay that measures nuclear DNA fragmentation. An assay for gene identification for an anti-proliferation pathway is the p15 induction assay. Wnt assay is a beta-catenin induction and/or translocation assay. STAT assay employs a readout cell line containing a reporter gene, under the control of any of STAT-inducible promoters. AP-1 is identified using a c-fos induction readout assay. CREB is identified using a phosphorylated readout assay. Nfk B translocation assay may be used to identify cells with nFkB translocation to the nucleus. For NFAT signaling pathway assay, a readout cell expressing a reporter gene such as CAT or luciferase under the control of an NFAT response element is used.